ANTITUMOR ACTIVITY OF DERIVATIVES OF MYCOPHENOLIC ACID

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One hundred and eight derivatives of mycophenolic acid (MA) have been prepared by modifications at the phenolic hydroxyl and/or carboxyl sites. None of these compounds was as effective as MA in suppressing cell growth of L-5178Y cell *in vitro*, whereas several compounds with changes at both the hydroxyl and carboxyl groups were more effective than MA against EHRLICH solid carcinoma and L-1210 leukemia in mice.

Mycophenolic acid, a metabolite of *Penicillium* first isolated in 1896, has been shown by us and other several groups to have antiviral, antitumor, antifungal and immunosuppressive properties. Studies on these activities of MA by our co-workers have been reported in this journal.[1,2,3,4]

MA has been reported active against several murine solid tumors and WALKER 256 in rat.[2,5,6] This antibiotic is relatively inactive against murine leukemias and ascites tumors but it is active against the ascitic form of the WALKER 256 tumor. Chemical transformation to a product with increased activity against murine leukemia may result in an increase in clinical usefulness of this unique antitumor agent. Previous attempts by others to synthesize more active MA derivatives have not been successful.[6,7] We now report several derivatives with increased activities.

Materials and Methods

Agents

All the agents tested in this paper were synthesized in our laboratories. For administration to mice, compounds were dissolved or suspended in saline or saline containing 0.25 % carboxymethylcellulose.

Antitumor activity against experimental tumor

To develop solid EHRLICH tumor (ESC), *ddy/S* male mice (4 weeks old) were inoculated subcutaneously with 5×10^6 EHRLICH ascites cells. L-1210 leukemia cells (1×10^6) were injected intraperitoneally into ($C_3H/He\times DBA/2$) hybrid mice. Minced X-5563 myeloma tumor (5 mg) was implanted subcutaneously into C_3H/He mice. Eighteen hours after inoculation with tumor cells intraperitoneal treatment was initiated and continued for 5 consecutive days with various dose levels of compound. Antitumor activity was evaluated by comparison of the tumor weights of control and treated mice with ESC and X-5563 tumors (10th and 12th days after implantation respectively) and the life-span in L-1210 leukemia mice.

Inhibition of the growth of L-5178Y cells *in vitro*

L-5178Y cells were grown at 37°C in CO_2-incubator in RPMI-1640 medium containing 10 % of calf serum. L-5178Y cells (4.0×10^4 cells in 0.9 ml medium) in the exponential phase of growth were preincubated in glass tubes for 20 minutes. After preincubation, 0.1 ml aliquots of derivatives were added per tube, and these were incubated for 48 hours. The number of cells were determined with hemocytometer. $TCID_{50}$ (50 % tissue culture inhibitory dose) of agent was obtained graphically.

Rf value of thin-layer chromatogram

To obtain some information about the correlation between the biological activities of the derivatives and their lipophilic character, Rf values of thin-layer chromatograms were determined by the method of HUSSAIN and LIEN,[8] using Eastman chromatogram silica gel thin-layer sheets and *n*-BuOH - AcOH - H_2O (4:2:1) as developing solvent. After development, the locations of the compounds were visualized by means of complexation with I_2 and Rf values were calculated.

Results and Discussion

MA has a low order inhibitory activity against both L-1210 and ESC (Table 1). We attempted to enhance the activity by chemical modification of this natural product.

It is of significance that all features of the MA structure are essential for retaining the inhibitory effect. Foregoing references[6,8] and our preliminary study revealed that the inhibitory activity was lost or decreased by conversion of MA structure. For example, reduction or oxidation of olefinic part, demethylation of methoxyl group, oxidation of methyl group attached to benzene nucleus into methylol and lengthening or shortening of the terpene side chain all resulted in disappearance of or decrease in antitumor activity.

In this investigation we attempted to convert the phenolic hydroxyl group and/or the carboxyl group into enzymatically labile functional groups, that could probably be converted to their original forms *in vivo*. That is, we intended to take a course of drug latentiation but not of drug design.

Table 1 indicates $TCID_{50}$ values of 108 derivatives against L-5178Y cells and T/C values against ESC and L-1210 at dosages of 150 mg/kg or in several exceptions at dosages of 75 or 37 mg/kg of test product.

Compounds 1 to 20 were prepared by modification of the phenolic hydroxyl group leaving the carboxyl group unchanged. Compounds 1 to 7 are acylated products. On the whole, the acylation of the hydroxyl group had no influence on the anti-L-1210 activity, but sometimes enhanced the anti-ESC activity (acetylation product 1). Acylation with higher alkanoic acid resulted in decrease of activity against both L-1210 and ESC. Compounds 8 to 20 are carbamoyl derivatives of the hydroxyl group. As with acylation, carbamoylation did not improve the anti-L-1210 activity but some compounds showed satisfactory effects against ESC.

Compounds 21 to 30 are carboxyl group modified products in which the phenolic hydroxyl is intact. Simple alkyl esters 21 to 23 had increased activity over MA against L-1210, but they had equal or lower activity against ESC. This tendency is the reverse of that observed in hydroxyl group modifications. Alkyl esters 24 to 26, in which alkyl groups are substituted by hetero-atom(s), did not show any improved effect against L-1210.

Because some compounds obtained by acylation or carbamoylation of the phenolic hydroxyl group have more intense activity against ESC than MA and in some cases the esterification of the carboxyl group enhanced the anti-L-1210 activity, we synthesized compounds 31 to 108 in which both the hydroxyl and carboxyl groups are derivatized.

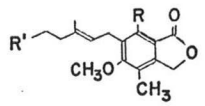
As was expected, compound 32 (the hydroxyl group was acetylated and the carboxyl group was converted into methyl ester) showed enhanced activity against both L-1210 and ESC. Similarly, simultaneous introduction of the functional groups which has been proven to be favorable in case of modification of the hydroxyl group alone or the carboxyl group alone,

respectively, showed improved activity against both of the two tumor types as in the case of compound 32. On the whole, simple alkyl esters enhanced activity but introduction of hetero-atoms into the alkyl groups (enhancement of hydrophilic character) decreased the activity, particularly against L-1210. This is in agreement with observations when only the carboxyl group was modified.

It appears that for optimization of activity there are structural limitations on the acyl or carbamoyl groups which are used for modification of the hydroxyl group. Acylation with simple aliphatic acids (formic, acetic and butyric acids) is advantageous but not with aromatic or hetero-atom-containing aliphatic acids (36 to 45). Carbamoylated $\left(-OCON\begin{matrix}R_1\\R_2\end{matrix}\right)$ compounds in which both of R_1 and R_2 are hydrogen (46 to 48) or one of them is hydrogen and another is phenyl, benzyl or cyclohexyl exhibited increased activity. Disubstituted carbamates other than compound 61 to 63 $\left(-OCON\begin{matrix}\diagup\\\diagdown\end{matrix}\!\!\bigcirc\right)$ showed no effect.

As seen in Table 1, none of these compounds was as effective as MA in suppressing growth of L-5178Y cells *in vitro*. However, some compounds showed better activity against ESC and/or L-1210 than MA *in vivo*. The correlations among three biological activities of these derivatives

Table 1. Antitumor activity of mycophenolic acid derivatives



Compound No.	R	R′	Antitumor activity		
			$TCID_{50}$[a] (mcg/ml)	L-1210[b] (T/C)	ESC[b] (T/C)
MA	$-OH$	$-COOH$	0.10	1.24	0.34
1	$-OCOCH_3$	$-COOH$	0.17	1.33	0.13
2	$-OCO(CH_2)_3CH_3$	$-COOH$	0.51	1.27	0.47
3	$-OCO(CH_2)_6CH_3$	$-COOH$	0.73	1.31*	0.47*
4	$-OCO(CH_2)_{10}CH_3$	$-COOH$	7.63	1.11*	1.00*
5	$-OCOCH_2Cl$	$-COOH$	0.23	1.28	0.20
6	$-OCOCH=CHCH_3$	$-COOH$	0.54	1.14	0.54
7	$-OSO_2CH_3$	$-COOH$	1.20	1.28	0.65
8	$-OCONH_2$	$-COOH$	0.11	1.01	0.44
9	$-OCON\begin{matrix}H\\\diagdown CH_3\end{matrix}$	$-COONa$	0.18	0.97	0.44
10	$-OCONH-\bigcirc$	$-COOH$	0.40	1.27	0.20
11	$-OCONH-\bigcirc-Cl$	$-COOH$	0.15	1.37	0.12
12	$-OCONH-\bigcirc\begin{matrix}CH_3\\CH_3\end{matrix}$	$-COOH$	0.27	1.36	0.18

Table 1. (continued)

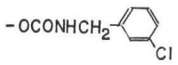
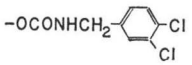
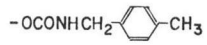
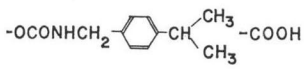
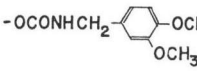
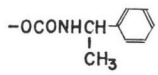
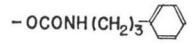
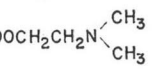
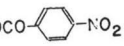
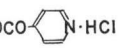
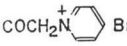
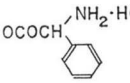
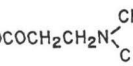
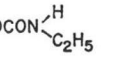
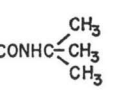
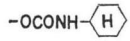
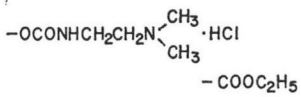
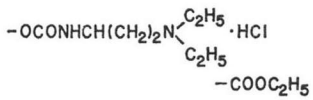
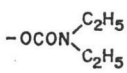
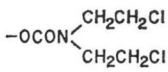
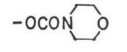
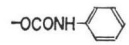
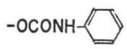
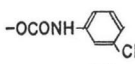
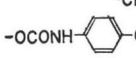
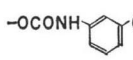
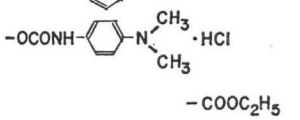
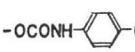
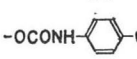
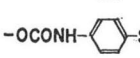
Compound No.	R	R′	Antitumor activity		
			$TCID_{50}$ (mcg/ml)	L-1210 (T/C)	ESC (T/C)
13		-COOH	0.10	1.39	0.06
14		-COOH	0.12	1.28	0.05
15		-COOH	0.16	1.33	0.13
16		-COOH	0.33	1.33	0.34
17		-COOH	0.22	1.22	0.20
18		-COOH	0.32	1.30	0.29
19		-COOH	0.23	1.20	0.20
20	-OCONHNHCSNH$_2$	-COOH	1.09	0.93	nt
21	-OH	-COOCH$_3$	0.18	1.43	0.68
22	-OH	-COOC$_2$H$_5$	0.40	1.38	0.34
23	-OH	-COOC$_4$H$_9$	0.72	1.38	0.46
24	-OH		0.20	nt	0.89
25	-OH		nt	1.00	0.72
26	-OH	-COOC$_2$H$_4$OH	0.12	1.25	0.28
27	-OH	-COOCH$_2$CH$_2$OCONH$_2$	0.3	1.35	0.19
28	-OH		1.2	1.00	0.99
29	-OH	-CONHCH$_2$COOH	nt	1.03	0.38
30	-OH	-CH$_2$OH	0.54	1.07	0.28
31	-OCHO	-COOCH$_3$	0.17	1.29	0.13
32	-OCOCH$_3$	-COOCH$_3$	0.28	1.50	0.18

Table 1. (continued)

Compound No.	R	R′	Antitumor activity		
			$TCID_{50}$ (mcg/ml)	L-1210 (T/C)	ESC (T/C)
33	$-OCOCH_3$	$-COOC_2H_5$	0.34	1.37	0.32
34	$-OCOC_7H_{15}$		0.55	1.33	0.55
35			0.23	1.18	1.27
36		$-COOCH_3$	0.69	1.19	0.69
37	$-OCOCH_2NH_2 \cdot HCl$	$-COOCH_2CH_2OH$	0.75	1.00	0.69
38		$-COOC_2H_5$	0.29	1.08*	0.27*
39		$-COOC_2H_5$	0.32	1.16	0.34
40		$-COOC_2H_5$	9.74	1.22*	1.00*
41	$-OCO(CH_2)_5NH_2 \cdot HCl$	$-COOC_2H_5$	14.0	0.85*	1.00*
42	$-OCOCH_2NH_2 \cdot HCl$	$-COOC_3H_7$	8.40	0.80	0.92
43	$-OCOCH_2NH_2 \cdot HCl$	$-COOC_2H_5$	0.24	1.18	0.24
44		$-COOC_2H_5$	1.92	1.16	0.54
45	$-OCO(CH_2)_4COONa$	$-COOC_2H_5$	0.92	1.07	0.68
46	$-OCONH_2$	$-COOCH_3$	0.20	1.56	0.20
47	$-OCONH_2$	$-COOC_2H_5$	0.29	1.47	0.10
48	$-OCONH_2$	$-COOC_4H_9$	0.54	1.45	0.14
49	$-OCONH_2$	$-COOC_2H_4OH$	0.18	1.18	0.23
50	$-OCONH_2$		0.26	1.18	0.23
51	$-OCONH_2$		1.8	1.01	1.12
52		$-COOCH_3$	0.17	1.15	0.27
53		$-COOC_2H_5$	0.12	1.23	0.17

Compound No.	R	R′	Antitumor activity		
			$TCID_{50}$ (mcg/ml)	L-1210 (T/C)	ESC (T/C)
54	$-OCONH(CH_2)_5CH_3$	$-COOC_2H_5$	0.34	1.15	0.33
55		$-COOC_2H_5$	0.19	1.57	0.13
56	$-OCONH(CH_2)_{11}CH_3$	$-COOC_2H_5$	0.31	1.37	0.20
57		$-COOC_2H_5$	0.25	1.23	0.47
58		$-COOC_2H_5$	0.70	1.14*	0.70*
59		$-COOC_2H_5$	0.24	1.18	0.21
60	$-OCONH(CH_2)_5COONa$	$-COOC_2H_5$	9.8	0.97	0.82
61		$-COOCH_3$	1.11	0.78	1.00
62		$-COOCH_3$	3.32	1.16	0.94
63		$-COOCH_3$	50	0.95	1.43
64		$-COOC_2H_5$	0.43	1.46	0.21
65		$-COOCH_3$	0.41	1.56	0.41
66		$-COOCH_3$	0.25	1.22	0.26
67		$-COOC_2H_5$	0.19	1.42*	0.39*
68		$-COOC_2H_5$	0.18	1.62	0.26
69		$-COOC_2H_5$	0.11	1.54	0.08
70		$-COOC_2H_5$	0.31	1.62	0.17
71		$-COOC_2H_5$	0.25	1.38	0.23
72		$-COOC_2H_5$	0.14	1.56	0.13
73		$-COOC_2H_5$	0.20	1.57	0.20
74		$-COOC_2H_5$	0.24	1.64	0.21

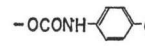
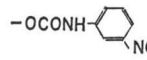
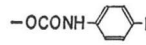
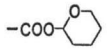
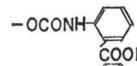
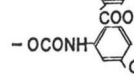
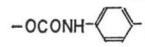
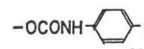
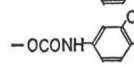
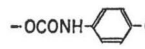
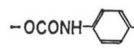
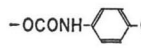
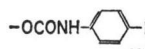
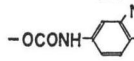
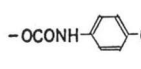
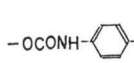
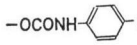
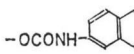
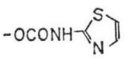
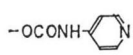
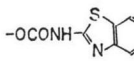
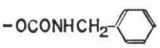
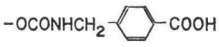
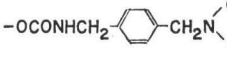
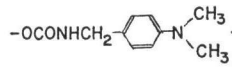
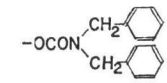
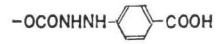
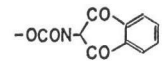
Compound No.	R	R′	Antitumor activity		
			$TCID_{50}$ (mcg/ml)	L-1210 (T/C)	ESC (T/C)
75		$-COOC_2H_5$	0.21	1.60	0.22
76		$-COOC_2H_5$	0.15	1.60	0.17
77			2.7	1.14	0.62
78		$-COOC_2H_5$	0.92	1.02	0.78
79		$-COOC_2H_5$	0.42	1.10	0.41
80		$-COOC_2H_5$	0.23	1.45	0.18
81		$-COOC_3H_9$	0.18	1.57	0.23
82		$-COOC_2H_5$	0.23	1.48	0.21
83		$-COOC_2H_5$	0.34	1.57	0.31
84		$-COOC_2H_5$	0.48	1.53	0.42
85		$-COOC_2H_5$	0.42	1.26	0.43
86		$-COOC_2H_5$	0.16	1.51	0.20
87		$-COOC_2H_5$	0.17	1.51	0.19
88		$-COOC_2H_5$	4.8	1.00	1.02
89		$-COOC_2H_5$	0.45	1.14	0.42
90		$-COOC_2H_5$	0.24	0.63	0.15
91	 Arginine salt	$-COOC_2H_5$	0.16	1.22	0.19
92		$-COOC_2H_5$	0.12	1.42	0.10
93		$-COOC_2H_5$	1.25	1.14	0.61
94		$-COOC_2H_5$	2.12	1.14**	0.79**
95		$-COOC_2H_5$	1.24	1.00	0.88

Table 1. (continued)

Compound No.	R	R′	Antitumor activity		
			$TCID_{50}$ (mcg/ml)	L-1210 (T/C)	ESC (T/C)
96		$-COOC_2H_5$	0.33	1.62	0.26
97		$-COOC_2H_5$	0.62	1.05	0.26
98		$-COOC_2H_5$	0.51	0.98	0.51
99		$-COOC_2H_5$	0.13	1.39	0.08
100		$-COOC_2H_5$	9.8	1.02	0.95
101	$-OCONHOH$	$-COOCH_3$	0.12	1.25	0.45
102	$-OCONHNHCSNH_2$		0.74	1.14	0.77
103		$-COOC_2H_5$	0.72	1.20	0.63
104		$-COOCH_3$	0.38	1.14	0.45
105	$-OCONHNHCSNH_2$	$-COOCH_3$	0.17	1.14	0.72
106			nt	1.09	0.52
107	$-OSO_2Na$	$-COOCH_3$	nt	1.18	1.02
108	$-OCONH_2$	$-CH_2OCONH_2$	1.6	1.05	0.98

a) $TCID_{50}$ value against L-5178Y cells *in vitro*.
b) The ratio of treated group/control group in average life-span (L-1210) or average tumor weight after 10 days after tumor inoculation (ESC) at the doses of 150 (not marked), 75 (*) and 37 mg/kg (**), respectively. (These compounds with marks were toxic at the dose of 150 or 75 mg/kg.)

and their lipophilic character are illustrated graphically. As shown in Figs. 1 and 2, $TCID_{50}$ values of these compounds were plotted against T/C values of ESC and L-1210. $TCID_{50}$ values of compounds corresponded more closely to activity against ESC than that against L-1210, *i.e.*, points are scattered more widely in Fig. 2 than in Fig. 1. It is apparently suggested for these tendencies that the activity of compounds against ESC is mainly dependent upon the activity *in vitro*, whereas the activity of compounds against L-1210 did not depend upon the activity *in vitro*, but was under the influence of other characteristics of the agent. It is notable that MA has the lowest $TCID_{50}$ value but its activities against ESC and L-1210 are moderate. This lower *in vivo* activity may be caused by glucuronidation and rapid excretion in urine.[9]

Correlation between Rf values and activities against ESC or L-1210 are shown in Figs. 3 and 4, respectively. As expected, all highly active compounds against L-1210 showed high Rf values. This characteristic was not observed in the activity against ESC. This fact suggests

Fig. 1. Correlation between $TCID_{50}$ value and activity against ESC.

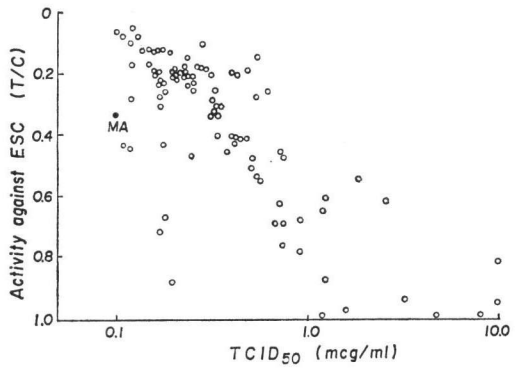


Fig. 2. Correlation between $TCID_{50}$ value and activity against L-1210.

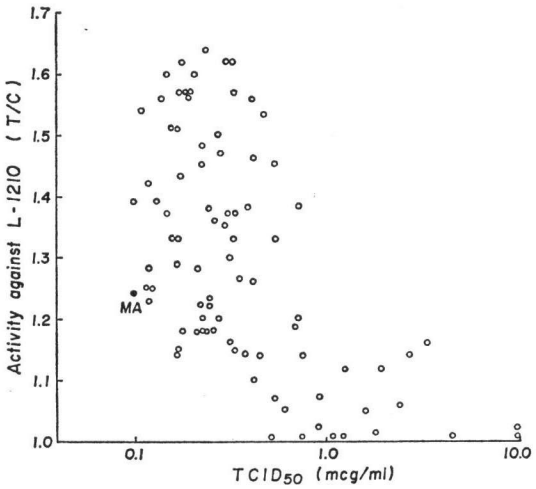


Fig. 3. Correlation between Rf value and activity against ESC.

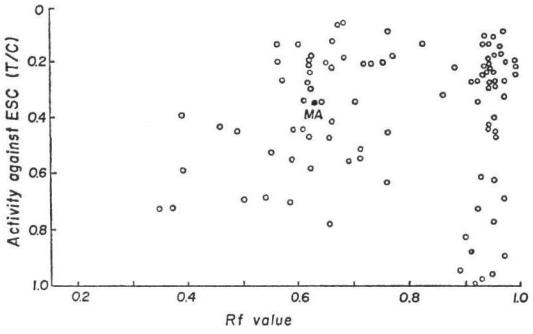
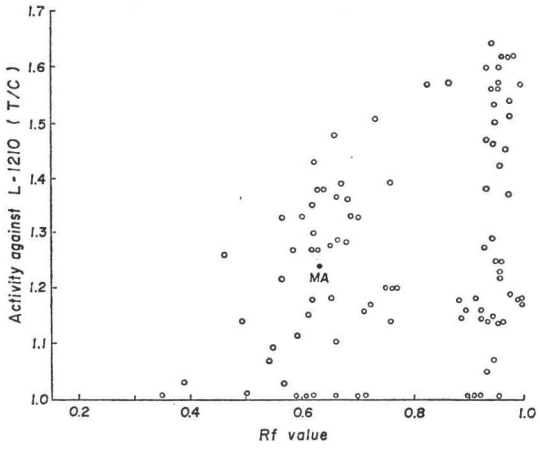


Fig. 4. Correlation between Rf value and activity against L-1210.



that the lipophilic character of the compound is an important factor on exhibiting the potential activity against L-1210 in mice.

In conclusion, these results, similar to observations with other drugs, indicate the hydrophilic and lipophilic balance (HLB) which influences absorption and distribution in a living body seems to be a very important factor. Esterification of the carboxyl group appeared to be advantageous in increasing the lipophilic character to an appropriate extent. However some structural limitations observed on the modification of hydroxyl groups can not be explained by HLB alone. Changes in activity may be subtly concerned with absorption and stability *in vivo*, affinity to tumor cells, behavior of fragments on enzymatic decomposition, and other factors.

The activity of compound 46 at various dosage levels against L-1210, ESC and X-5563 were shown in Tables 2, 3 and 4, respectively. In these tumor system, compound 46 showed better curative effects than MA.

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Table 2. Comparative antitumor activity of compound 46 and MA against L-1210.

Compound	Dose* (mg/kg)	Av. survival day	T/C	Body wt. change** (g)
46	500	13.8	1.86	−5.0
	300	12.2	1.65	−3.8
	150	11.2	1.51	−0.8
	50	9.6	1.30	1.4
	15	9.0	1.22	2.2
MA	500	9.6	1.30	0.6
	300	9.6	1.30	0.5
	150	8.8	1.19	0.4
	50	8.6	1.16	1.4
	15	8.2	1.11	2.0
Control		7.4	1.00	3.7

* Injected intraperitoneally daily for 5 days.
** Difference in the body weight before and at 6 days after tumor inoculation.

Table 3. Comparative antitumor activity of compound 46 and MA against ESC.

Compound	Dose* (mg/kg)	Av. tumor wt.** (mg)	TC %	Survival
46	300	36.8	6.0	6/6
	150	124.8	20.3	6/6
	50	301.0	49.5	6/6
	15	413.6	67.3	6/6
MA	300	144.4	23.5	6/6
	150	180.0	29.4	6/6
	50	333.8	54.3	6/6
	15	463.6	75.5	6/6
Control		613.7	100.0	6/6

* Injected intraperitoneally daily for 5 days.
** Tumors were weighed 10 days after tumor inoculation.

Table 4. Antitumor activity of compound 46 and MA against X-5563.

Compound	Dose* (mg/kg)	Av. tumor wt.** (mg)	T/C %	Survival
46	300	0	0	10/10
	150	22.8	1.1	10/10
	50	1,146.6	56.5	10/10
MA	150	267.8	13.1	10/10
	50	1,708.4	84.2	10/10
Control		2,029.0	100.0	10/10

* Injected intraperitoneally daily for 5 days.
** Tumors were weighed 12 days after tumor inoculation.

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